

ABSTRACT

Title of thesis: PRODUCTION AND CHARACTERIZATION OF NEW ALLELES
 OF SCAR IN DROSOPHILA MELANOGASTER

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Germline cysts are clusters of germ cells that arise by division from a single cell and are connected to one another by stable intercellular bridges. Cysts are a highly conserved stage of pre-meiotic germ cell development, but the molecular mechanisms that control their formation are highly unknown. Developing cysts in *Drosophila* contain a cytoplasmic organelle called a fusome, which plays a critical role in cyst formation. The *SCAR* gene, which encodes an actin regulatory protein, was identified in a screen for mutations that disrupt fusomes and cyst formation. It was not clear, however, if the mutation *l(2)k03107* disrupts only *SCAR*. To address this question, new alleles were created by excising the P-element in *l(2)k03107*. These alleles were then characterized molecularly and phenotypically. The results show that two alleles have deletions in the *SCAR* gene. Further testing must be done to determine the role of *SCAR* in cyst formation.

PRODUCTION AND CHARACTERIZATION OF NEW ALLELES OF SCAR IN
DROSOPHILA MELANOGASTER

by

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Chapter 1: Background

The major goal of this research project was to determine the role of *SCAR* in cyst and fusome formation in *Drosophila*. Cysts are small clusters of germ cells that can be found in phylogenetically diverse organisms (1). Germline cysts form when dividing germ cells do not complete cytokinesis (2). The daughter cells are linked via ring canals or intercellular bridges (2). The ring canals are organized in such a way that the interconnections are maximally branched (3). The number of cells in a cyst is usually 2^n , where n is a number from 1 to 8 and represents the number of divisions.

Cysts play an important role in gamete development (2). In males, the gametes develop in closely synchronized clusters (2). Male gametes differentiate after meiosis and contain different genomes. The gene products are then shared through the ring canals of the developing cyst. In females, however, the developing cyst has a different function (2). Developing oocytes complete most of their development prior to meiosis, and therefore remain genetically homogeneous. The developing oocytes become enormous in size and accumulate materials that are going to be stored for future use. As a result 15 out of the 16 cystocytes become nurse cells in *Drosophila* (2). The 16th cell becomes the oocyte. In most fly species, the oocyte develops from one of the two cells that has the most ring canals. This implies that there are polarizing mechanisms that determine which cell becomes the oocyte and which does not.

Cysts contain a large cytoplasmic organelle known as the fusome (2). A small, spherical fusome can be found in stem cells and cystoblasts. A cystoblast is the

cell that divides to form a cyst. In dividing cysts, however, it is large and branched. At the end of the first division of the cystoblast, the fusome remains in only one of the two daughter cells because the spindle orients with one pole in the fusome (4). New fusomal material then accumulates in the new ring canal. Next, the cystoblast divides again, generating four cells. Once again, the spindle orients with one pole near the fusome and the fusome remains in the two central cells. New fusomal material again accumulates in the new ring canals. This process repeats itself with each new division, leading to branched fusomes (2). The fusome gradually breaks down and disappears as the oocyte in 16-cell cysts forms (2). Fusomes can also be found in males, but do not break down prior to meiosis. Lin et. al. identified two components of the fusome (6). They found that the fusome is composed of the membrane skeletal proteins α -spectrin and Hu-li tai shao (Hts) (6). Hts is a homolog of adducin (7). The fusome also contains ankyrin and β -spectrin (8). It also contains actin (deCuevas unpublished data). Fusomes are important for proper cyst formation and oocyte determination (9). Mutants that have defective fusomes also have defects in some aspects of cyst formation and are therefore unable to produce gametes that are functional. Mutations in the genes encoding α -spectrin and Hts lead to the disruption or elimination of the fusome (8, 6). These mutations result in the formation of cysts that lack an oocyte and contain fewer than 16 cells (8, 7). Hts and α -spectrin mutations that eliminate the fusome greatly reduce the number of cystocytes in germline cysts and severely affect oocyte determination (9). Without the fusome, cystocytes divide asynchronously at a greatly reduced frequency and with random spindle orientations, suggesting the important role of the fusome in cyst

formation (9). The fusome is also important for organizing a polarized microtubule network for the intracyst transport of specific molecules that lead to oocyte differentiation (9). The fusome is asymmetrically distributed in cysts and it is thought that the cell that inherits the fusome becomes the oocyte (5). In addition, mutations that disrupt the fusome also disrupt synchrony, as indicated by the production of cysts that no longer contain 2^n cells. Grieder et. al. also describe a function of the fusome. They state that the fusome interacts with and organizes the microtubule network during late interphase in both mitotic and meiotic cysts (10).

As stated earlier, fusomes are full of actin (de Cuevas and Spradling, unpublished data). A protein that regulates actin polymerization is SCAR (2). SCAR is a suppressor of cAR, a G-protein coupled receptor (11). SCAR is also a regulator of the Arp2/3 complex. The Arp2/3 complex is activated by Scar/WAVE and Wiskott-Aldrich Syndrome protein (WASp). It stimulates the rate-limiting step in actin polymerization, microfilament nucleation. The Arp2/3 complex is a complex of seven proteins (12). The seven proteins are the actin-related proteins Arp2 and Arp3, arpc1, and four novel proteins (13). The Arp2/3 complex, in addition to having nucleation activity, mediates the formation of dynamic branched actin networks at the leading edge of motile cells by binding to the sides of preexisting actin filaments (12).

The Arp2/3 complex can also regulate actin structures that are not involved in motility (12). For example, during oogenesis in *Drosophila* defects in the actin-lined ring canals can be attributed to the loss of the Arp2/3 complex. At the periphery of each ring canal there is a layer of actin filaments attached to the plasma membrane (14). This actin

lining is organized into loosely packed antiparallel bundles. Arp2/3 and Scar mutant egg chambers have a “dumpless” phenotype—the nurse cells fail to transport their cytoplasmic contents to the oocyte (15). The Arp2/3 complex is important for remodeling the actin cytoskeleton in vivo and slows the spontaneous rate of actin filament nucleation in vitro (13). The Arp2/3 complex has many other functions. It is involved in the growth of ring canals during oogenesis, furrow formation divisions of the syncytial blastoderm, the formation of the central nervous system, and the morphogenesis of the eye and sensory bristles (15).

Zallen et. al. studied SCAR in depth. They produced mutant alleles of *SCAR* using the P element *l(2)k13811* (16). They produced imprecise excisions of *SCAR*^{*k13811*}. All of these except one took out part of the neighboring gene *l(2)06225*. These excisions were homozygous lethal and did not complement the lethality of *SCAR*^{*k13811*}. According to Zallen et. al., *SCAR*^{*k13811*} mutants have mutant central nervous system axon morphology (16). Zallen et. al. also generated an imprecise excision of *SCAR* known as *SCAR*^{*Δ37*} (16). In *SCAR*^{*Δ37*} both *SCAR* and part of the neighboring gene *piwi* have been removed (16). According to Cox et. al., removing PIWI decreases the rate of division of germline stem cells (17). Therefore, PIWI is a promoter of germline stem cell division (17). Taking out part of *piwi* makes it difficult to look at this mutation in early oogenesis. Zallen et. al. generated clones using the FLP/FRT system and found that in *SCAR*^{*Δ37*} mutants, the egg chamber has a multinucleate phenotype as opposed to individual cells (16). In addition, the ring canals are smaller in size and the eggs are abnormally shaped and small (16). These results suggest that *SCAR* plays an important role in oogenesis.

Chapter 2: Specific Aim

SCAR is also disrupted by the P element *l(2)k03107* (16). A screen was performed and identified *SCAR*^{*k03107*} as a mutant that disrupts the fusome and cyst formation (deCuevas and Fasnacht, unpublished data). The fusome in *SCAR*^{*k03107*} mutants is not branched like wild type fusomes (deCuevas and Fasnacht, unpublished data). *SCAR*^{*k03107*} is a more severe mutant than *SCAR*^{*Δ37*}, a null allele of *SCAR* that technically should not be less severe than *SCAR*^{*k03107*}. There are several possible explanations for this discrepancy. One possibility is perdurance. Some *SCAR* protein may have still been present in the genetic clones that were generated by Zallen et. al. Another possibility is that *SCAR*^{*k03107*} is a gain-of-function allele. The specific aim of this project is to produce and characterize new alleles of *SCAR* by imprecise excision of the P element *l(2)k03107* in *Drosophila melanogaster* that do not take out the neighboring genes *piwi* and the uncharacterized *l(2)06225*. These new alleles will not contain the P element. This research should help to determine why *SCAR*^{*k03107*} is a more severe mutant and what role, if any, *SCAR* is playing in cyst development and fusome formation.

Chapter 3: Experimental Design and Methods

The purpose of the first set of crosses was to generate new alleles of *SCAR*. After the new alleles were generated they were characterized. It was determined if the excision of *SCAR* also removed part of the neighboring genes *l(2)k06225* and *fs(2)piwi*⁰⁶⁸⁴³. The alleles were also be characterized physically and molecularly to determine what portion of the gene has been removed.

Generation of New Alleles of *SCAR*:

To generate new alleles of *SCAR* using the P element *l(2)k03107*, the following reversion crossing scheme was performed.

$$\text{Cross 1: virgin } yw; \frac{\Delta 2-3Cy}{BcEgfr} \times \text{male } \frac{w}{Y}; \frac{l(2)k03107}{CyO}$$

In this cross the $\Delta 2-3Cy$ was the source of the transposase needed to excise the P element.

$$\text{Cross 2: male } \frac{yw}{Y}; \frac{l(2)k03107^{Ex?}}{\Delta 2-3Cy} \times \text{virgin } w; \frac{Sco}{CyO}$$

The purpose of cross 2 was to remove the source of the transposase, preventing the P element from moving around any further.

$$\text{Cross 3: male } \frac{yw}{Y}; \frac{l(2)k03107^{Ex?}}{CyO} \times \text{virgin } w; \frac{l(2)k03107}{CyO}$$

The purpose of this cross was to determine the type of excision—precise or imprecise. If the excision were precise, then $\frac{l(2)k03107^{Ex?}}{l(2)k03107}$ survived and straight-winged flies were produced. If the excision were imprecise, then $\frac{l(2)k03107^{Ex?}}{l(2)k03107}$ did not survive and all of the progeny of this cross had curly wings.

Cross 4: male $\frac{yw}{Y}$; $\frac{l(2)k03107^{Ex?}}{Cyo}$ x virgin w; $\frac{l(2)k03107^{Ex?}}{Cyo}$

This cross was the stock of the new alleles.

Determining if the excision of *SCAR* also removed part of the neighboring genes *l(2)k06225* and *fs(2)piwi*⁰⁶⁸⁴³:

The next set of crosses that were performed determined if the excisions also removed the neighboring gene *piwi*.

Cross 1: male $\frac{l(2)k03107^{Ex?}}{Cyo}$ x virgin $\frac{fs(2)piwi^{06843}}{Cyo}$

Cross 2: virgin $\frac{l(2)k03107^{Ex?}}{fs(2)piwi^{06843}}$ x any male

If $\frac{l(2)k03107^{Ex?}}{fs(2)piwi^{06843}}$ females were fertile, then the excision of *l(2)k03107* must not

have taken out part of *fs(2)piwi*⁰⁶⁴⁸³.

The next cross that was performed determined if *l(2)06225* was removed in addition to *SCAR*.

$$\text{male } \frac{l(2)k03107^{Ex?}}{CyO} \times \text{virgin } \frac{l(2)k06225cn}{CyO}; ry$$

If $\frac{l(2)k03107^{Ex?}}{l(2)06225}$ survived, then the excision of *l(2)k03107* did not take out part of *l(2)06225*.

Control Crosses:

To make sure that the experiment is working correctly the following control crosses were also performed. First $\frac{l(2)k03107}{CyO}$ was crossed to $\frac{l(2)06225cn}{CyO}$ and $\frac{fs(2)piwi^{06843}}{CyO}$.

$\frac{l(2)06225}{l(2)k03107}$ should have survived and $\frac{l(2)k03107}{fs(2)piwi^{06843}}$ females should have been fertile.

Next $\frac{SCAR^{l(2)k13811}FRT40A}{Cy \text{ Roi } cn \text{ bw}}$ was crossed to $\frac{l(2)06225cn}{CyO}$ and $\frac{fs(2)piwi^{06843}}{CyO}$.

$\frac{SCAR^{l(2)k13811}FRT40A}{l(2)06225}$ should have survived and $\frac{SCAR^{l(2)k13811}FRT40A}{fs(2)piwi^{06843}}$ females should

have been fertile. Then $\frac{SCAR^{\Delta 37}FRT40A}{CyO \text{ En} - lacZ}$ was also crossed to $\frac{l(2)06225cn}{CyO}$ and

$\frac{fs(2)piwi^{06843}}{CyO}$. $\frac{SCAR^{\Delta 37}FRT40A}{l(2)06225}$ should have survived and $\frac{SCAR^{\Delta 37}FRT40A}{fs(2)piwi^{06843}}$

females should have been sterile. Next, $\frac{SCAR^{l(2)k13811}FRT40A}{Cy \text{ Roi } cn \text{ bw}}$ was crossed to

$\frac{l(2)k03107}{CyO} \cdot \frac{SCAR^{l(2)k13811} FRT40A}{l(2)k03107}$ should have survived. Finally,

 $\frac{SCAR^{\Delta 37} FRT40A}{CyO \text{ } En - lacZ}$ was crossed to $\frac{l(2)k03107}{CyO} \cdot \frac{SCAR^{\Delta 37} FRT40A}{l(2)k03107}$ should not have

 survived.

Molecular Characterization:

Next DNA was made from heterozygous flies using the QIAmp DNA mini kit from Qiagen. PCR was carried out using standard protocols on a Biometra T Gradient thermocycler. The PCR products were then run out on a gel and purified from gel fragments using the QIAquick gel extraction kit from Qiagen. The fragments were then sequenced at the Center for Biosystems Research sequencing facility. The location of the primers in the genome is depicted in Figure 1.

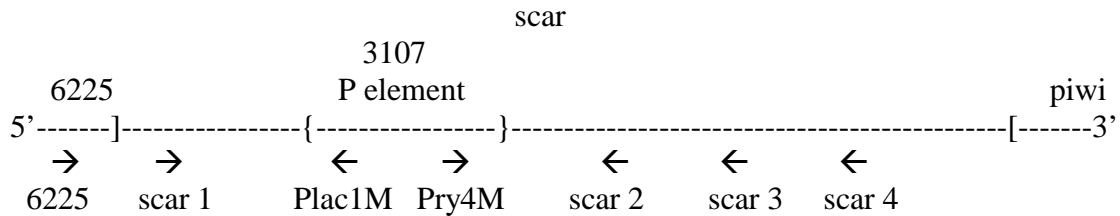


Figure 1: The location of the PCR primers

Phenotypic Characterization:

Next, two of the new alleles were characterized phenotypically using the FLP/FRT system. First, two of the imprecise excisions were crossed to $\frac{FRT40A}{Cyo}$. In the second

cross of this crossing scheme virgin $\frac{l(2)K03107^{Ex}}{FRT40A}$ was crossed to $\frac{Sco}{Cyo}$. A

recombination event occurred at this step that placed the FRT on the same chromosome

as the imprecise excision. Next, $\frac{l(2)k03017^{Ex} FRT40A}{Cyo}$ was crossed to *hs flp; GFP*

FRT40A. Heat shocking $\frac{l(2)k03107^{Ex} FRT40A}{FRT40AGFP}$ resulted in a recombination event that

led to the formation of homozygous mutant clones. The ovaries of these clones were then dissected, stained with DAPI, a nuclear stain, and viewed under the fluorescent microscope.

Chapter 4: Results

Of the 77 new alleles that were generated, 31 were precise excisions and 46 were imprecise. Two of the imprecise excisions did not remove $fs(2)piwi^{06483}$ or $l(2)06225$.

$\frac{l(2)06225}{l(2)k03107}$ survived and $\frac{l(2)k03107}{fs(2)piwi^{06843}}$ females were fertile. $\frac{SCAR^{l(2)k13811}FRT40A}{l(2)06225}$

should survive and $\frac{SCAR^{l(2)k13811}FRT40A}{fs(2)piwi^{06843}}$ females should be fertile. $\frac{SCAR^{\Delta 37}FRT40A}{l(2)06225}$

survived and $\frac{SCAR^{\Delta 37}FRT40A}{fs(2)piwi^{06843}}$ females were sterile. $\frac{SCAR^{l(2)k13811}FRT40A}{l(2)k03107}$ survived.

$\frac{SCAR^{\Delta 37}FRT40A}{l(2)k03107}$ survived even though it theoretically should not have survived. There

is currently no explanation for this discrepancy. PCR results revealed that for the excision R7 only part of the P element, the $w+$ gene, was removed. The ends of the P element remained intact (Figure 2).



Figure 2: PCR reaction #1, Lane 1: standard, Lane 2: 3107 DNA with primers scar 1 and Plac1M, Lane 3: R7 DNA with primers scar 1 and Plac1M, Lane 4: 3107 DNA with primers Pry4M and scar 2, Lane 5: R7 DNA with primers Pry4M and scar 2, Lane 6: wildtype DNA with primers 6225 and scar 2, Lane 7: R7 DNA with primers 6225 and scar 2, Lane 8: wildtype DNA with primers scar 1 and scar 2, Lane 9: R7 DNA with primers scar 1 and scar 2, Lane 10: wildtype DNA with primers scar 1 and scar 3, Lane 11: R7 DNA with primers scar 1 and scar 3.

Similar results were seen with excision R6. However, the entire P element is missing in excisions R14 and R17 (Figure 3).



Figure 3: PCR reaction #2. Lane 1 contains the standard. Lanes 2-5 contain DNA with primers scar 1 and Plac1M. The DNA in Lane 2 is wildtype, Lane 3=3107, Lane 4=R6, Lane 5=R14, and Lane 6=R17. Lanes 7-11 contain DNA with the primers Pry4M and scar 2. The DNA in Lane 7 is wildtype, Lane 8=3107, Lane 9=R6, Lane 10=R14, and Lane 11=R17.

Figure 4 shows that the entire P element is missing in excisions R14 and R17.

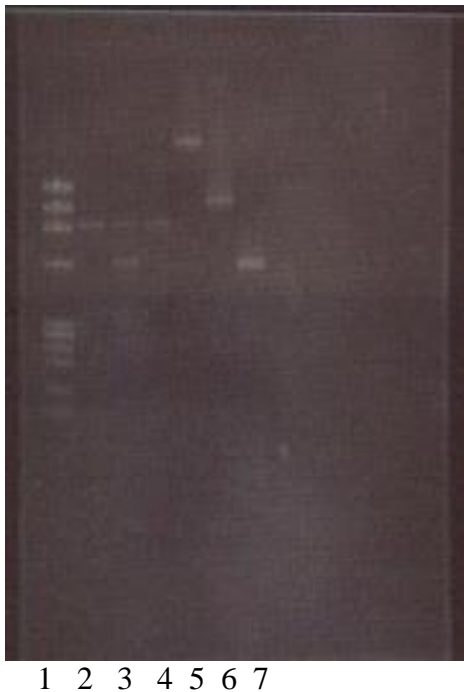


Figure 4: PCR reaction #3, Lane 1: standard, Lane 2: wildtype DNA with primers 6225 and scar 3, Lane 3: R14 DNA with primers 6225 and scar 3, Lane 4: R17 DNA with primers 6225 and scar 3, Lane 5: wildtype DNA with primers scar 1 and scar 4, Lane 6: R14 DNA with primers scar 1 and scar 4, Lane 7: R17 DNA with primers scar 1 and scar 4.

Next, the PCR product of R14 with primers scar 1 and scar 4 was sequenced using primer scar 1 (Figure 4). According to this sequence, part of the 5' untranslated region (UTR), all of intron 1, ATG, and the beginning of the coding sequence were removed from *SCAR*.

TTTCN**Δ**CCAAGCTTCGGTCACACTGCAAAGGTGTGGAAATCTTAG**GT**TTCCACC
ATGATGAAATAACATGTTATTATAACGCGCATCGATCGCCTAGCTATCAAAG
TTACCCAGCTGGACAGCACAGTTGAGGAGGTGCCCTTGACGGACATTACCCG
AAAGAAGGCCTTCAAGTCGGCCAAGGTTTTTGATCAACAGATCTTCTCGCGC
GCCACCATGCCGGCACCAATGATGGACACATATGCCCAGTGCGACAAACCGC
CGCCCCTCGACAAGCTTAATGTCTATCGAGACGATGGCAAGGATGGTCTTAA
GTTCTACACGGATCCGAACTACTTCTTTGAGTTGTGGCGACAGGAGATGCTGA
 AGGATACCGAACGTGTGATGCACGACAAGGGCAAGAAGCTGAACAGACCGC
 GGCAGGATGGCGGTGCCGGCGGAGCTGCTGGACGTGGCAATAAGAAACAGA
 AGACCAAAATAAGGGTGCCGCACAATACGCGCGAACAGCAGCGACAACGTG
 CACTGGTGCACGGCGAAACATTGATGCCAAACAACGTCATCTACCGCACACC
 GAATTCCATGGTCAACGAAGAAGCCGGATACGGAAGTAAGTACTTGGCGAA
 AGGGATTGGTCTCCGTGTGAGCCAAGTATTCAGTGCGAACCAAAATTCATAGG
 CTGTTATTGGTACGTTTCGTTTCGCGCACTGACTATTGCACTTGTCTTGCCTATT
 GAATGGTACATCTATTTTAATTATCAATTATGTCCTGTCTGCCCGTGTAGTTCA
 GCTAGTCAAAATACAGACTGTGGGCGAGGCTGACATCGATTTGCGGCATAGC
 ACTCTCGATGCGCACCAAGTTGTTCGGTAATATCCAAGACGTAGGTACGA
 TGATGGGGCCNCGTAGCTTTCATTTCCC

Figure 5: Sequence of R14

Key: **Outline Underlined** is the start of the 5' UTR, Outline Underlined is the original P-element insertion site (between GT) within the 5' UTR, Dotted Underlined is the 5' end of P-lacW, Wavy Underlined is the Scar 3 primer

Next, the PCR product of R17 with primers scar 1 and scar 4 was sequenced using primer scar 1 (Figure 5). According to this sequence, part of the 5' untranslated region (UTR), all of intron 1, and most of the first exon (including primer scar 3) were removed from *SCAR*.

CN**ACCAAGCTTCGGTCACACTGCAAAGGTGTGGAAATCTTAGTTTC**CGTTTCC
GGATACGGAAGTAAGTACTTGGCGAAAGGGATTGGTCTCCGTGTGAGCCAAG
 TATTCAGTGCGAACCAAATTCATAGGCTGTTATTGGTACGTTTCGTTTCGCGCA
 CTGACTATTTGCACTTGTCTTGCCTATTGAATGGTACATCTATTTTAATTATCA
 ATTATGTCCTGTCTGCCCGTGTAGTTCAGCTAGTCAAAATACAGACTGTGGGC
 GAGGCTGACATCGATTTGCGGCATAGCACTCTCGATGCGCACCACCAAGTTG
 TCGGTAATATCCAAGACGTAGGTACGATGATGGGGCCACGTAGCTTTCATTTTC
 CGCCCGGCAGACATCCACGATACAGATGTACCCGCCATTCATTCACCTACTT
 CACTCAAAAGCTCGCTAGTTTTTCGATGCGCCTCAAAACCCAGTTCGAAGATC
 ACTAATTTGTATTATTTTGAAGTTGCTATTAATTGAAATTAACCCACACAATC
 ATGACCCAGAGAAAGGTGAANNNCNNNANCNNNNNNNCNNA

Figure 6: Sequence of R17

Key: **Outline** is the start of transcription which ends two base pairs 5' to the site of P-element insertion, Outline Underlined is the end of transcription, Dotted Underlined is what is left of exon 1, Wavy Underlined is the scar 4 primer

The FLP/FRT system revealed that the imprecise excisions R14 and R17 did not have any defects in the ovarioles.

Chapter 5: Conclusion

The major goal of this project was to produce and characterize new alleles of *SCAR* in *Drosophila melanogaster*. Two new alleles, R14 and R17, were generated which delete significant portions of the *SCAR* gene. However, these alleles did not have the expected phenotype. Further testing needs to be done to determine the role of *SCAR* in cyst formation. The study of cyst formation in this model system should contribute to the understanding of cyst formation in more complex animals such as humans.

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